

Optimization of elm regeneration *in vitro* using leaf explants and evaluation of the process in transformation experiments

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Abstract

We set up a regeneration process for the elm hybrid 'Sapporo' resistant to Dutch Elm Disease, using leaf discs from *in vitro* grown plants and tested it in transformation experiments. Two steps were needed : bud induction (3 weeks) followed by bud elongation (4 weeks). Bud meristems initiated near the cut ribs, from several tissues (phloem, procambium, rib upper parenchyma) except epidermis. Regeneration depended on the use of agarose (6 g/l LSM) and diluted MS medium (1/2). The best results (11-14 buds/explant) were obtained in presence of 0.1 μM TDZ and 0.06 μM IAA and when maltose (55-110 mM) or sorbitol (110 mM) were used as carbohydrate sources. During shoot elongation TDZ should be decreased to 0.01 μM or replaced by 1-2 μM BAP and 1.4 μM of GA3 added. At this stage, a mixture of 55 mM sorbitol and 27.5 mM maltose was required. Up to 7 shoots / explant developed and were easily rooted (96%) and acclimatized when sorbitol (27.5 mM) and active charcoal (2 g/l) were added to the rooting medium. Transformation experiments were performed with *Agrobacterium tumefaciens* disarmed strain GV3101-pMP90 (pKyGUS-intron). Regeneration zones exhibited stable GUS expression. However, all shoots grown *in vitro* died on a selective rooting medium (50 mg/l kanamycin). When attempts for *in vitro* selection were made (12.5 mg/l kanamycin), some buds were initiated but elongation was prevented. Susceptibility to neomycin seemed very high, therefore, selection markers and selection steps must be revised carefully.

Key words: *Ulmus*, 'Sapporo gold 2', *in vitro*, culture medium, GUS expression, neomycine sensitivity.

Resumen

Optimización de la regeneración *in vitro* del olmo utilizando explantes de hoja y evaluación del proceso en experimentos de transformación

Se ha desarrollado un procedimiento para la regeneración del olmo híbrido «Sapporo» resistente a la grafiosis mediante el uso de discos foliares procedentes de plantas cultivadas *in vitro* y examinadas en ensayos de transformación. Para ello se requirieron dos pasos: inducción de las yemas (3 semanas), seguido de la elongación de las yemas (4 semanas). Los meristemas que dieron origen a las yemas se formaron en la cercanía de las costillas de corte a partir de diversos tejidos (floema, procámbium, parénquima) excepto la epidermis. La regeneración dependió del uso de agarosa (6 g/l LSM) y de medio MS diluido (1/2). Los mejores resultados (11-14 yemas por explante) se obtuvieron en presencia de 0,1 μM de TDZ y 0,06 μM de IAA y cuando la maltosa (55-110 mM) o el sorbitol (110 mM) se usaron como fuente de carbohidratos. Durante la elongación del tallo, el TDZ hubo de ser disminuido a 0,01 μM o sustituido por 1-2 μM de BAP y 1,4 μM de GA3. En esta fase fue necesaria una mezcla de 55 mM de sorbitol y 27,5 mM de maltosa. Se desarrollaron al menos 7 tallos por explante que fácilmente enraizaron (96%) y se aclimataron cuando se añadió sorbitol (27,5 mM) y carbón activo (2 g/l) al medio de enraizamiento. Los ensayos de transformación se desarrollaron con las cepas GV3101-pMP90 (pKyGUSintron) de *Agrobacterium tumefaciens*. Las zonas de regeneración presentaron una expresión GUS estable. No obstante, todas las yemas cultivadas *in vitro* murieron en un medio selectivo de enraizamiento (50 mg/l de kanamicina). Cuando se intentó la selección *in vitro* (12,5 mg/l de kanamicina), algunas yemas se iniciaron pero la elongación fue impedida. La susceptibilidad a la neomicina parece ser muy alta, por lo que los marcadores y los pasos de selección deben ser cuidadosamente revisados.

Palabras clave: *Ulmus*, 'Sapporo gold 2', *in vitro*, medio de cultivo, expresión GUS, sensibilidad a la neomicina.

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Introduction

Genetic transformation should be a useful tool for elm preservation and improvement. First it could be used to introduce either Dutch Elm Disease (DED) or bark beetle resistance genes (Sticklen *et al.*, 1994). Second it could be used to introduce marker genes that could make easier the selection of somatic hybrids after protoplast fusion as it was done for *Solanum tuberosum* (Masson *et al.*, 1988). Somatic hybridization was often used within *Solanaceae* to transfer bacterial or fungal resistance (Jarl *et al.*, 1999; Fock *et al.*, 2000) and provide a useful alternative to conventional breeding. Whatever the purpose, efficient and reliable plant regeneration systems must be set up. European elm capacity to regenerate *in vitro*, from cambium cells, is well known since the very beginning of *in vitro* culture (Gautheret, 1940a and 1940b; Jacquot, 1949, 1951, 1955). More recently, *in vitro* plant regeneration has been obtained from several explants (leaves, internodes, callus, isolated cells and protoplasts) and has been reported for *U. americana* (Bolyard *et al.*, 1991; Cheng *et al.*, 1992, Durzan and Lopushanski, 1975; George and Tripepi, 1994; Ulrich *et al.*, 1984), *U. procera* (Dorion *et al.*, 1991; Fenning *et al.*, 1993), *U. parvifolia* (Bolyard *et al.*, 1991), *U. pumila* (Kapaun and Cheng, 1997) and hybrids like *U. x 'Pionner'* (Fink *et al.*, 1986; Sticklen *et al.*, 1986), *U. x 'Commelin'* (Dorion *et al.*, 1995) and *U. x 'Dodoens'* (Dorion *et al.*, 1994). Although plant regeneration was successful, efficiency remained often too low.

We have chosen to work with two hybrids, one susceptible to DED [*'Commelin'*, (*U. Glabra* Huds. x *U. carpinifolia*) x *U. carpinifolia*, Heybroek 1993] and the other one resistant [*'Sapporo autumn gold 2'*, *U. pumila* L. x *U. japonica* (Redh.) Sarg., Smalley and Lester, 1973] since these are suitable for somatic hybridization. In order to set up efficient regeneration systems, we firstly worked with cambium explants (Ben Jouira *et al.*, 1997, 1998). The efficiency of the process depended on the season and on the genotype. The results were interesting for *'Commelin'* (20 buds/explant, 7 rootable shoots) but were quite low for *'Sapporo gold'* (6-7 buds/explant, 3 rootable shoots). Moreover, with this method we were not able to get transformed plants (Ben Jouira *et al.*, 2000a). After coculture with an *Agrobacterium tumefaciens* engineered strain, cambium explants produced green shoots on a kanamycin containing medium (250 mg/l). However, isolated shoots did not root on selective me-

dium (50 mg/l kanamycin). GUS test and PCR analysis with *uidA* primers were negative.

Therefore, to improve both *'Sapporo'* plant regeneration and transformation efficiency, we tried to use leaf discs. We report here on the improvement of *'Sapporo'* plant regeneration using leaf explants and testing the process for transformation experiments.

Material and Methods

Plant material

'Sapporo' was micropropagated *in vitro* by subculturing rooted pinched shoot every 4-5 weeks (Dorion *et al.*, 1987). The medium contained DKW (Driver and Kuniyuki, 1984) macronutrients x 1/4, 2.5 µM indolbutyric acid (IBA), 2 g/l activated charcoal and 6 g/l agarose LSM®. The first three unfolded leaves were excised from the shoots and used for regeneration experiments. Stem apices, rooted via a 1-week auxinic chock (15 µM IBA without activated charcoal) were used for the renewal of mother plant.

In vitro culture

Leaf discs (8 mm diameter, 2 discs/leaf), taken across the midvein, were cultured on induction medium for 3 weeks (6 discs/90 mm x 16 mm Petri dish), and subcultured on elongation medium for 4 weeks (100 mm x 25 mm Petri dish).

The induction and elongation media contained macronutrients MS x 1/2 or 1/4 (Murashige and Skoog, 1962), or DKW complete medium or diluted (x 1/2 or 1/4), micronutrients of Heller (1953), Fe-citrate (160 µM Fe), vitamin mixture of Morel and Wetmore (1951), carbon sources (27.5, 55 or 110 mM maltose, sucrose, glucose, fructose or sorbitol), IAA (0, 0.06, 0.6 µM) and TDZ (0.1 µM). TDZ (0.1, 0.01 µM), Benzylaminopurine (BAP, 1 and 2 µM) and Gibberellic acid (GA3, 0.14 and 1.4 µM) were specifically tested, at several concentrations, during the elongation stage. Two type of agar were also tested: 8 g/l Bitek agar (DIFCO) and 4-7 g/l agarose (LSM). The pH was adjusted to 5.5 and stabilized with Methyl ethyl sulfonic acid (MES buffer, 3.5 mM) before autoclaving (112°C, 5 MPa, 20 min).

Adventitious shoots (5-8 mm) were excised and rooted in test tubes on a rooting medium containing

All explants were kept in growth chamber at 25°C under 16 h of fluorescent light (43 $\mu\text{mol m}^{-2} \text{s}^{-1}$), 22°C (at dark). Rooted plantlets were acclimatized in the greenhouse according to Dorion *et al.* (1987).

Leaf discs were fixed in FAA (5 ml formaldehyde, 5 ml acetic acid, 90 ml ethanol 50°) and dehydrated in ethanol baths (50°-100°). They were then embedded in paraffin (PF 56°C, Prolabo-France) and 8 µm serial transverse cuts were made using a rotary microtome. Sections were mounted on glass slides, then paraffin was removed with histolemon (Carlo Erba) baths and sections were hydrated in ethanol baths (100°-50°) and water. Safranin was used to stain nuclei, suberin and lignin while fast green was used to stain cellulose. Finally, sections were dehydrated in ethanol and histolemon and embedded in Canada balsam.

A specific plasmid vector pKYGUSintron (Fig. 1) was constructed and introduced into 2 diarmed *A. tumefaciens* strains GV3101pMP90 and LBA4404 by triparental conjugation. It contained the *nptII* gene conferring resistance to neomycins, and the *uidA* (*gus*) gene with an intron as reporter gene. This gene is under the control of the CaMV 35S promoter with a double enhancer sequence to increase GUS expression level.

Transformation experiments were conducted only with GV3101-pMP90 (pKYGUSintron) since it was shown that it is less inhibitory on the budding process (Ben Jouira *et al.*, 2000a). At excision time, leaf discs were put in bacterial suspension (20 min in 2.10^7 bacteria /ml), blotted on sterile paper and transferred to the regeneration medium. According to Ben Jouira *et al.* (2000a), a 3-4 days cocultivation period was applied and decontamination was performed with ticarcillin in liquid medium (200 mg/l for 5 min). Explants were then transferred, every two weeks, to fresh medium containing ticarcillin (200 mg/l). The GUS expression was assayed histochemically (Jefferson *et al.*, 1984) 1-5 weeks after the beginning of the treatment with *A. tumefaciens*. Me-

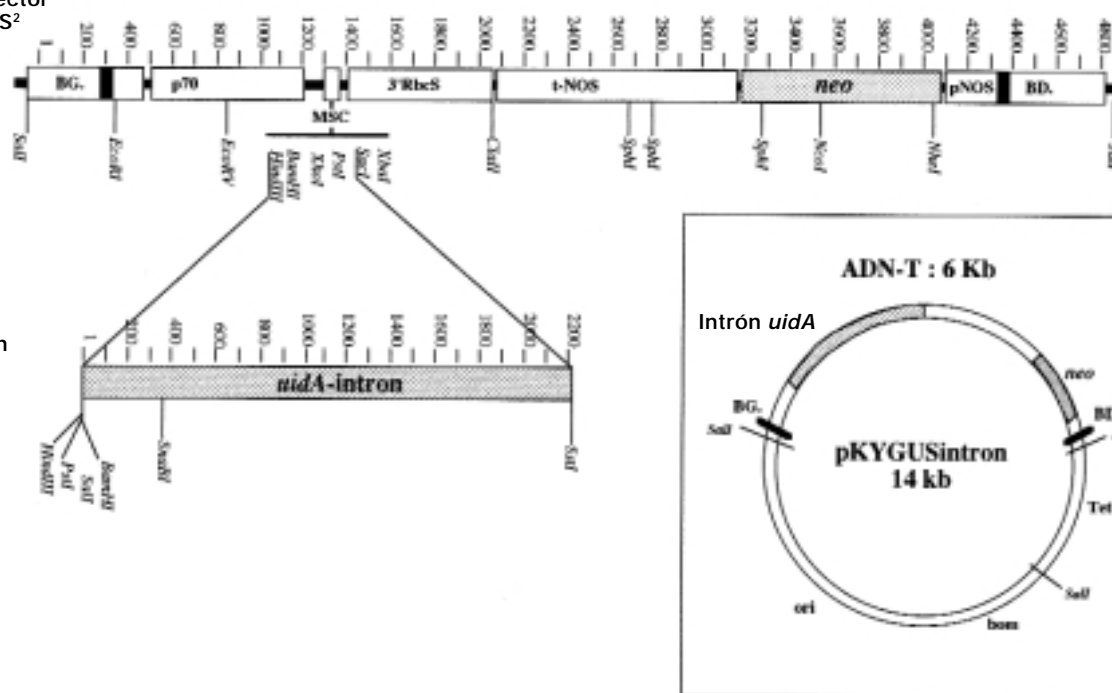


Figure 1. pKYGUSintron binary vector. BG: T-DNA left border. p70: promoter of the CAMV 35S-RNA with a double enhancer. PLK: polylinkers. 3'RbcS: Rubisco terminator. t-NOS: Nopaline synthase terminator. BD: T-DNA right border. ori: replication origin in *E. coli*. bom: mobilization site. TetR: tetracycline resistance gene.

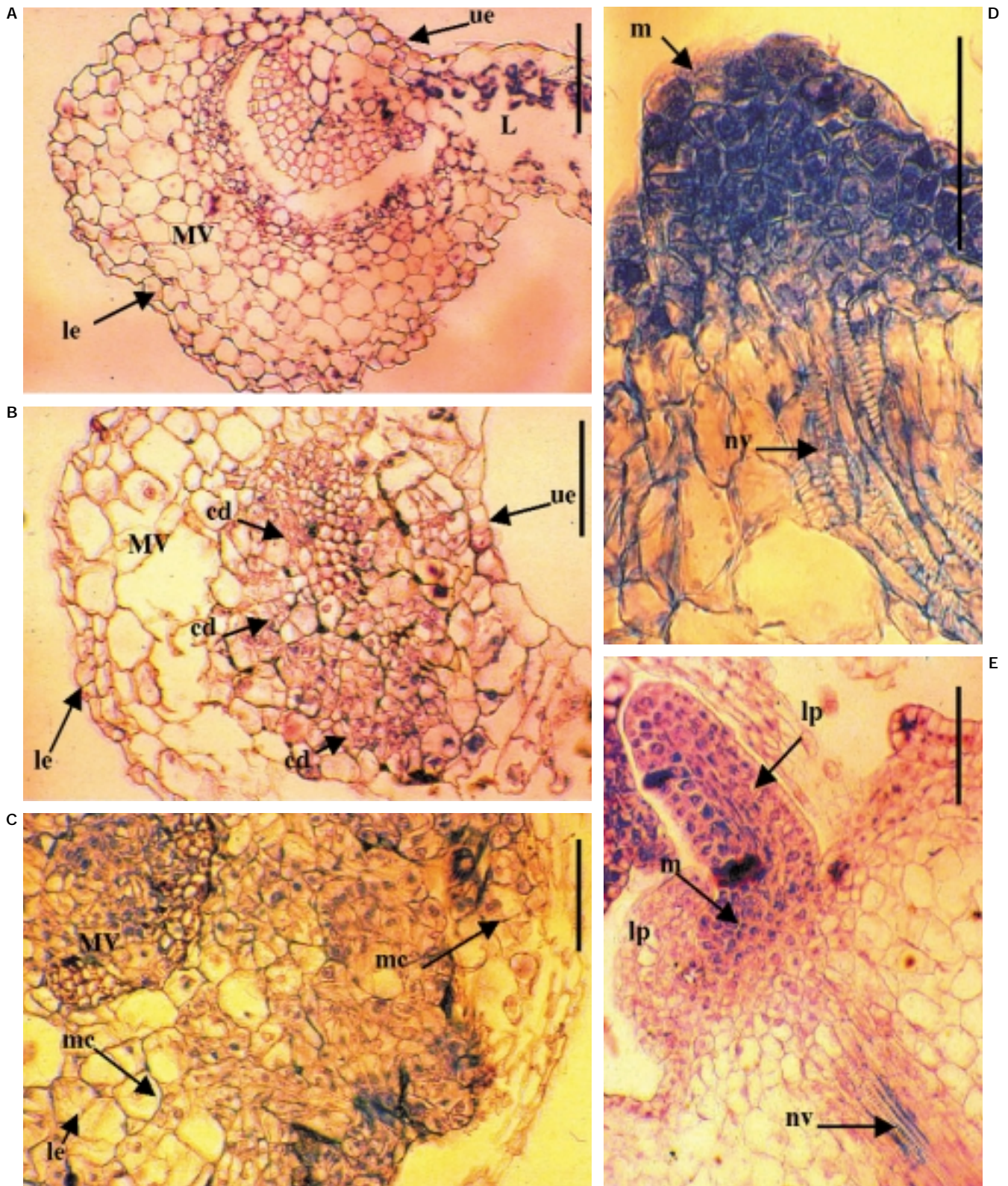


Figure 2. Histology of bud induction in 'Sapporo' elm leaf discs in the midvein region. a: 0 day. b: after 3 days. c: after 7 days. d: after 14 days. e: after 21 days. Bar = 100 µm. cd: cell division. L: lamina. le: lower epidermis. lp: leaf primordium. m: meristem. mc: meristematic dividing cells. MV: midvein. nv: new vessels. ue: upper epidermis.

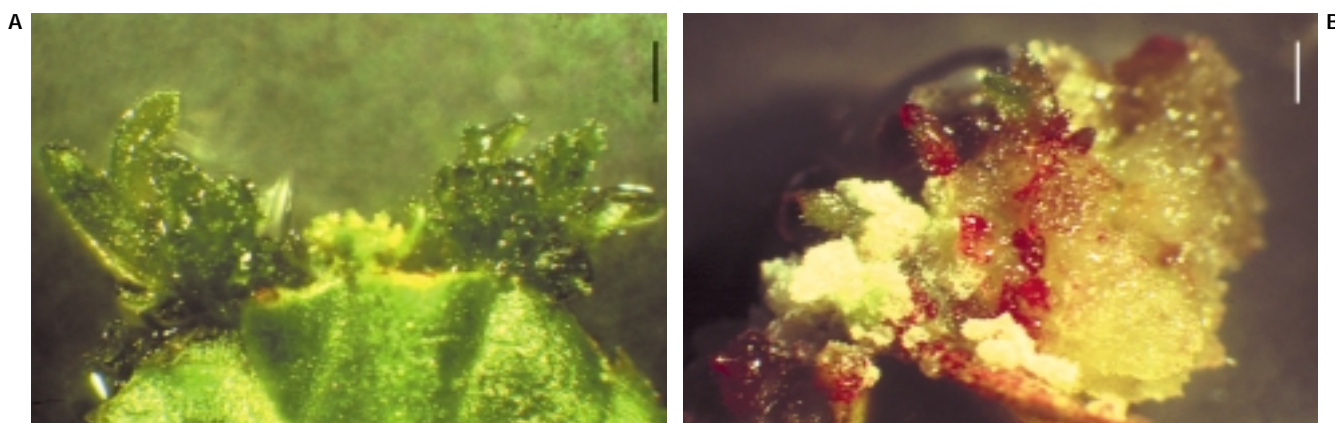


Figure 3. 'Sapporo' elm leaf disc bud induction after 3 weeks. Bar = 1mm. a: with 110 mM sorbitol. b: with 110 mM glucose.

thanol 20% was added to X-Gluc medium (1 mM) in order to suppress any endogenous - glucuronidase activity (Kosugi *et al.*, 1990). For the selection of transgenic plants, kanamycin and paromomycin were added to leaf disc culture medium (12.5, 25, 50, 75, 100, 150 mg/l). Kanamycin (50 mg/l) was added to the rooting medium to control plantlet resistance to this antibiotic.

Data collection and statistics

To evaluate regeneration, data were recorded after 3, 4 and 7 weeks and expressed as the percentage of explants with adventitious buds (regeneration frequency) and as the mean number of buds (meristem with 1-2 leaf initials) or shoots (5-8 mm) formed per responding explant. For rooting evaluation, data were recorded after 4 weeks and plant survival in greenhouse after 1 month.

Statistical tests were performed using the computer statistic program INSTAT (GraphPad, San Diego, USA). Data were submitted to variance analysis and Newman-Keuls test ($p = 0.05$), with variable transformation when needed. Proportions were compared using a Chisquare test of independence ($p = 0.05$). Some other data were given with the mean confidence interval to show data dispersion.

Results

Regeneration process

Mitotic activity began within 3 days (Fig. 2a, b), mainly in the phloem tissue and in the subepidermal cell layers of the rib upper part. Meristematic clusters

were formed after 7 days but neither the epidermis nor the parenchyma cells were involved (Fig. 2c). After 2 weeks, callus developed at the cut edges of the ribs and exhibited meristematic zones on the upper part some of them showing vascularization (Fig. 2d). After 3 weeks, some meristems produced 1-2 leaf primordia (Fig. 2e and 3a) and had a well developed vascular connection (Fig. 2e). Intensity of callus formation depended greatly of sugar used (Fig. 3a, b).

Bud induction

The combination 0.06 μM IAA + 0.1 μM TDZ was found satisfactory for 'Sapporo' bud induction (Table 1).

For 'Sapporo', whatever the macronutrients tested, regeneration frequency was about 90-100%, but the number of buds/explant was significantly larger with diluted MS ($\times 1/2$) (Fig. 4).

All carbohydrate sources allowed bud neoformation. However, glucose (110 mM) and sucrose were clearly less efficient (Fig. 5). Moreover, the largest number of buds was obtained with sorbitol (110 mM) and maltose (55 and 110 mM) since they gave 10-14 buds/ex-

Table 1. Effect of IAA associated with 0.1 μM TDZ on regeneration from leaf discs of 'Sapporo' (4 weeks of culture on a medium containing 110 mM glucose, 12 explants/treatment)

IAA (μM)	Regeneration efficiency (%)	Number of buds/explant
0.00	8.3	3.0
0.06	58.3	6.4 ± 4.1
0.60	8.3	2.0

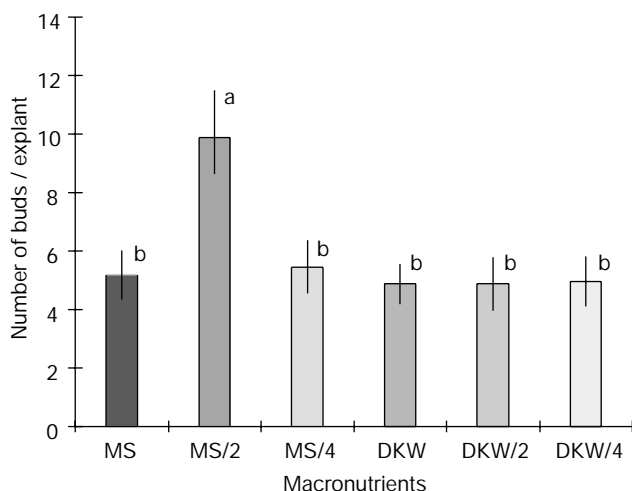


Figure 4. Influence of macronutrients on bud regeneration in 'Sapporo' elm leaf discs (24 explants/treatment) after four weeks on a medium containing 110 mM sorbitol; values with a different letter differed significantly ($p = 0.05\%$); bars indicate confidence limits.

plant while only up to 6 buds/explant were obtained in the other treatments (Fig. 6).

The use of agarose instead of agar significantly affected the number of buds per explant (Fig. 7), while the regeneration frequency was unaffected (87%).

Shoot growth

The beneficial effect of TDZ at $0.01\mu\text{M}$, and BAP at 1 and $2\mu\text{M}$ was significant (Fig. 8). When added to

the medium, GA_3 improved shoot growth (Table 2). Sorbitol was not as efficient for bud elongation than for bud induction (Fig. 9a) and addition of another sugar was required (Table 2, Fig 9a and b). When buds were initiated on maltose, no sugar modification was needed, except in the case of a half concentration (Fig. 9c and 9d).

Rooting and greenhouse acclimatization

Roots were observed one week after transfer to rooting medium. The use of diluted DKW macronutrient ($\times 1/4$) instead of MS ($\times 1/4$) improved significantly the rooting step from 58% to 96% ($p = 0.05$). Sorbitol at 27.5 mM favoured rooting (Table 3). Activated charcoal mainly decreased basal callus development, but associated with sorbitol promoted shoot growth (Table 3). More than 100 plants were acclimatized and their survival reached 100% when sorbitol was used in the rooting medium instead of sucrose (92%).

Transformation experiments

GUS expression was observed only on explants co-cultivated with *Agrobacterium* strain. It was detectable after 1 and 3 weeks on almost all the leaf discs (92-100%). After 1 week, the GUS expression was localized around the leaf disc for 50 % of the explants (Fig. 10a) and more intensely near the cut rib. After

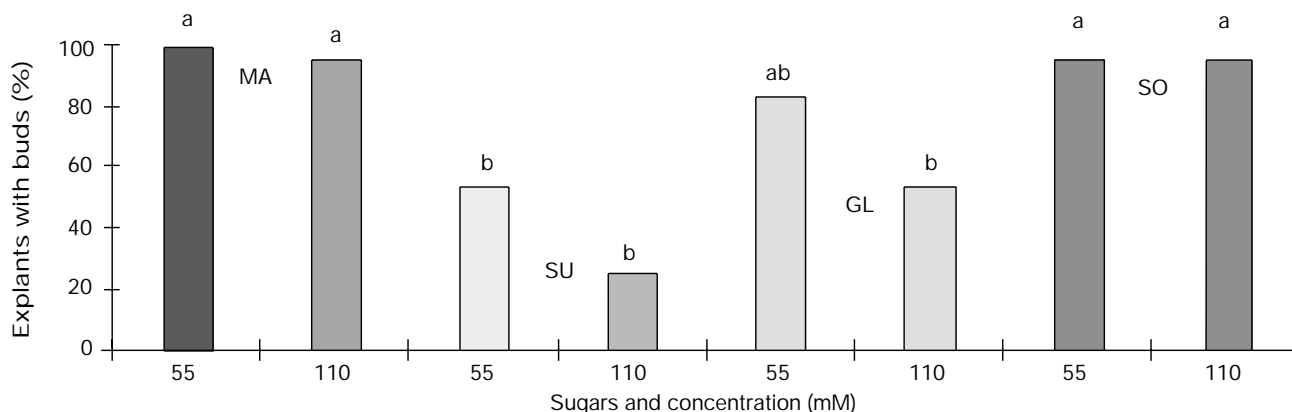


Figure 5. Influence of sugars (MA: mannitol. SU: sucrose. GL: glucose. SO: sorbitol) at different concentration on regeneration efficiency of 'Sapporo' elm leaf discs after 3 weeks (24 explants/treatment, results with the same letters are not significantly different $p = 0.05$); values with a different letter differed significantly ($p = 0.05\%$)

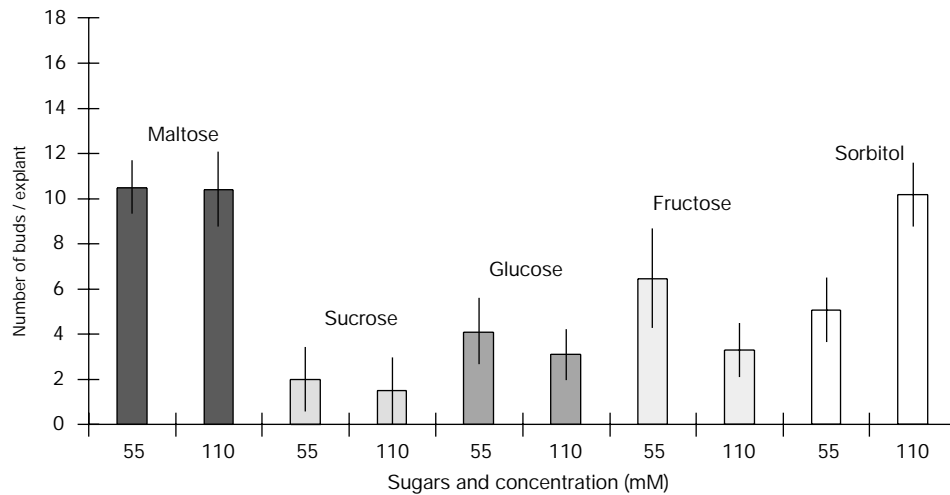


Figure 6. Influence of some sugars at different concentrations on bud formation from 'Sapporo' elm leaf discs after 3 weeks (24 explants/treatment, results were given with the confidence limits).

3 weeks, only the cut rib zone was stained (Fig. 10b), in which the buds arose (Fig. 3a). After 5 weeks, GUS expression decreased and was observed in only 25% of the explants, but always in the regeneration zone where some meristems were stained (Fig. 10c).

The regeneration process was very sensitive to kanamycin and paromomycin since budding was inhibited whatever the neomycin concentrations tested. However, in one experiment using 12.5 mg/l kanamycin, some buds were observed (Fig. 10d), but elongation was inhibited. Explants co-cultivated with *Agrobacterium* without kanamycin gave shoots almost normally.

Forty eight were subcultured on selective rooting medium (kanamycin 50 mg/l), but all of them died.

Discussion

The regeneration process described for 'Sapporo' leaf explants is very efficient and allows the production of 7 rootable shoots per explant (or 14 per leaf) within 7 weeks. This yield was higher than that obtained from small «cambium» explants for the same cultivar (Ben Jouira *et al.*, 1997). The same process ap-

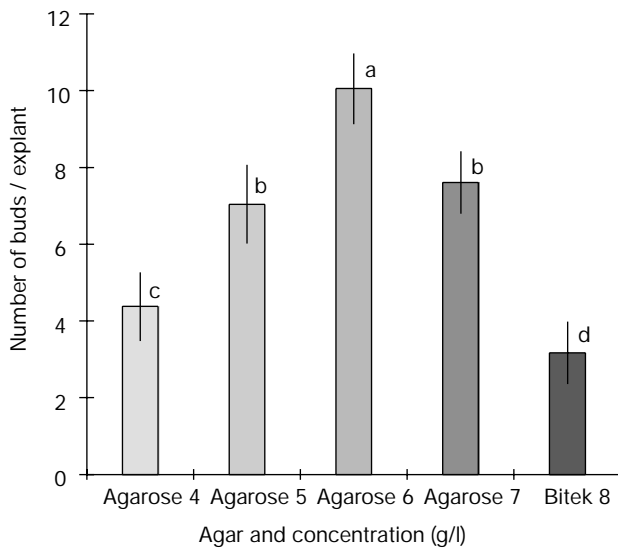


Figure 7. Effect of agar on bud formation from 'Sapporo' elm leaf discs after 4 weeks (24 explants/treatment); values with a different letter differed significantly ($p = 0.05\%$).

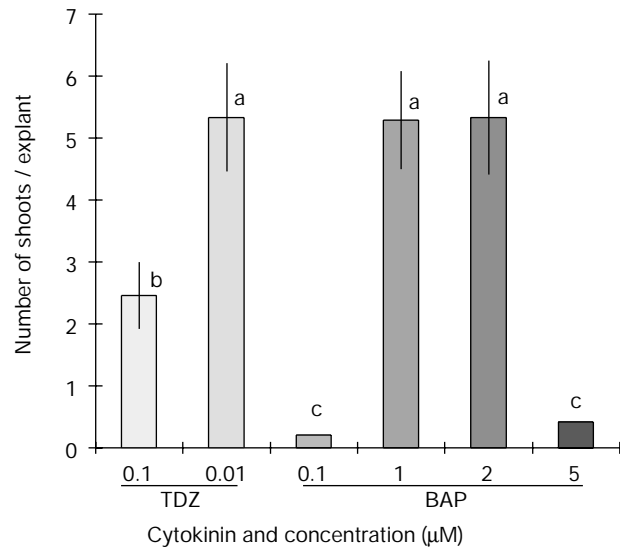


Figure 8. Effect of TDZ and BAP on adventitious bud formation from 'Sapporo' leaf discs after 7 weeks. (24 explants/treatment); values with a different letter differed significantly ($p = 0.05\%$).

Table 2. Influence of gibberellic acid GA₃ and sugars on shoot production from leaf disc of 'Sapporo' elm (number of shoots/explant \pm confidence interval) after 7 weeks of culture (24 explants/treatment)

GA ₃ (μ M)	Sorbitol (55 mM)	Sorbitol (55 mM) + Glucose (27.5 mM)	Mean ^a
0.00	0.6 \pm 0.3	1.6 \pm 0.7	1.1b
0.14	2.2 \pm 0.7	3.8 \pm 1.4	3.0a
1.40	2.3 \pm 0.8	5.5 \pm 1.1	3.9a
Mean ^a	1.7b	3.7a	

^a Values with different letter differed significantly ($p = 0.05\%$).

plied to 'Commelin' gave similar results, as reported before (Ben Jouira *et al.*, 2000b). Since these two hybrids originated from 4 species of elms (*U. capini-folia*, *U. glabra*, *U. pumila* and *U. japonica*) it is likely that the protocol will be efficient with any other elm genotypes, providing little adjustments.

For the two genotypes, we used micropropagated plants and a two step regeneration system. The use of agarose instead of standard agar had a major influence on regeneration. This could be due to a higher purity of the agarose compound. It is known that agar could release minerals, up to the toxic level, in culture medium (Debergh, 1983). We have shown the importance of a

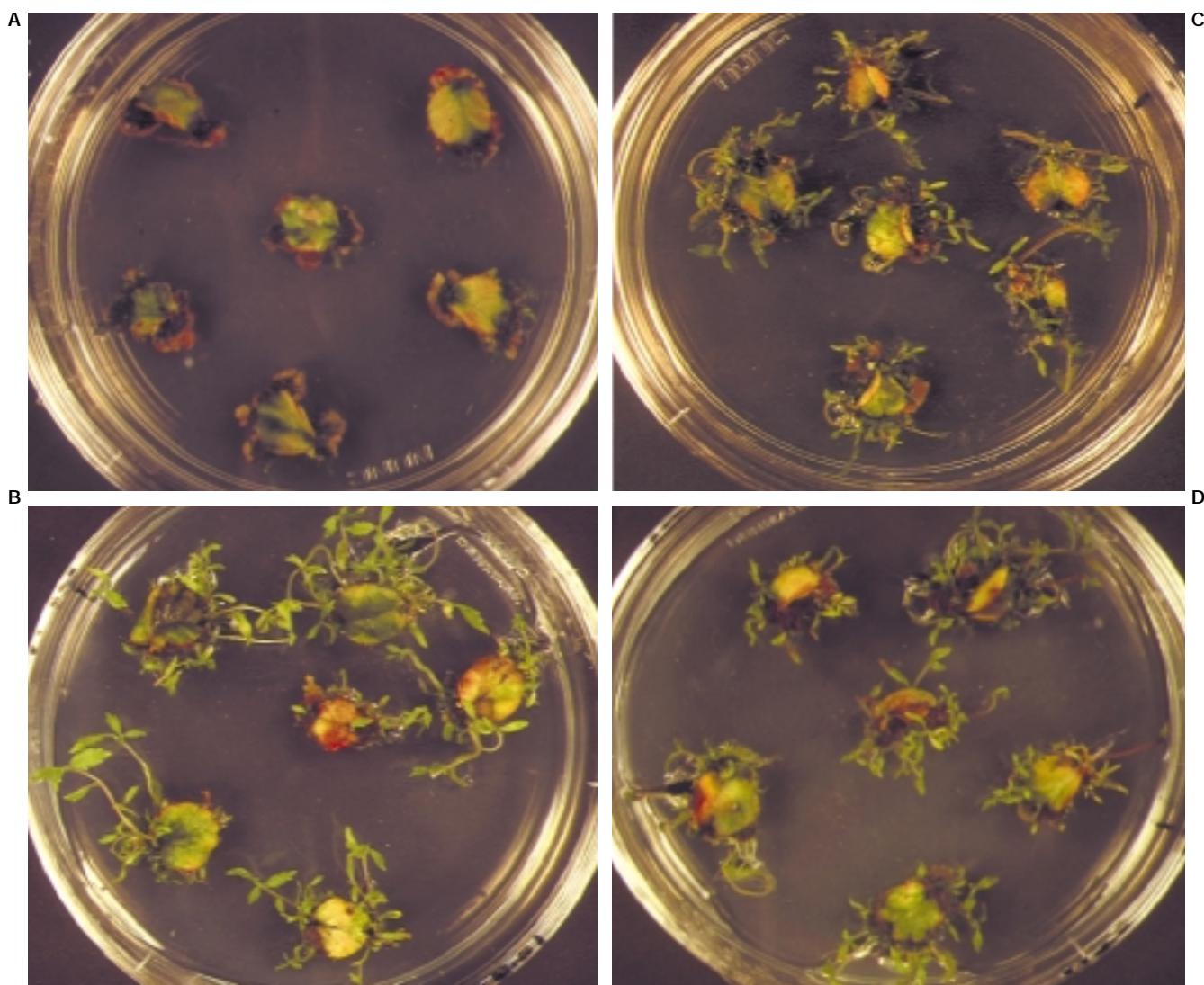


Figure 9. Effect of sugar sequences on adventitious bud formation from 'Sapporo' elm leaf discs (8 mm diameter) after 7 weeks. Bar = 1 cm. a: induction with 110 mM sorbitol and elongation with 55 mM sorbitol. b: induction with 110 mM sorbitol and elongation with 55 mM sorbitol + 27.5 mM maltose. c: induction with 55 mM maltose and elongation with 27.5 mM maltose. d: induction with 110 mM maltose and elongation with 55 mM maltose.

Table 3. Influence of sugars (27.5 mM) and active charcoal (2g/l) on rooting and growth of shoots regenerated from leaf explants of 'Sapporo' elm after 4 weeks of culture (24 shoots/treatment).

	Sucrose and charcoal ^a	Sucrose	Sorbitol and charcoal	Sorbitol
Rooting (%)	50 b	58 b	96 a	96 a
Number of roots/shoot	2.6 b	2.9 b	6.0 a	6.7 a
Callus diameter (mm)	1.7 b	3.0 a	1.4 b	2.7 a
Shoot length (mm)	3.8 b	3.3 b	4.4 a	3.5 b

^a Values with different letter within each file differed significantly ($p=0.05\%$).

low concentration of TDZ (0.1 μM for bud induction and 0.01 μM for elongation) although other authors (Bolyard *et al.*, 1991; George and Tripepi, 1994) found higher concentration more efficient (0.1 μM -22.5 μM). The effect of sugars is rarely studied on *in vitro* woody

plants. Generally, only sucrose is used at 60-90 mM. However, Kouider *et al.* (1984), Korban and Skirvin (1985) and Jordan *et al.* (1991) mentioned the efficiency of glucose on *Malus communis* and *Annona cherimola*. Conversely, sugar importance in the regeneration pro-

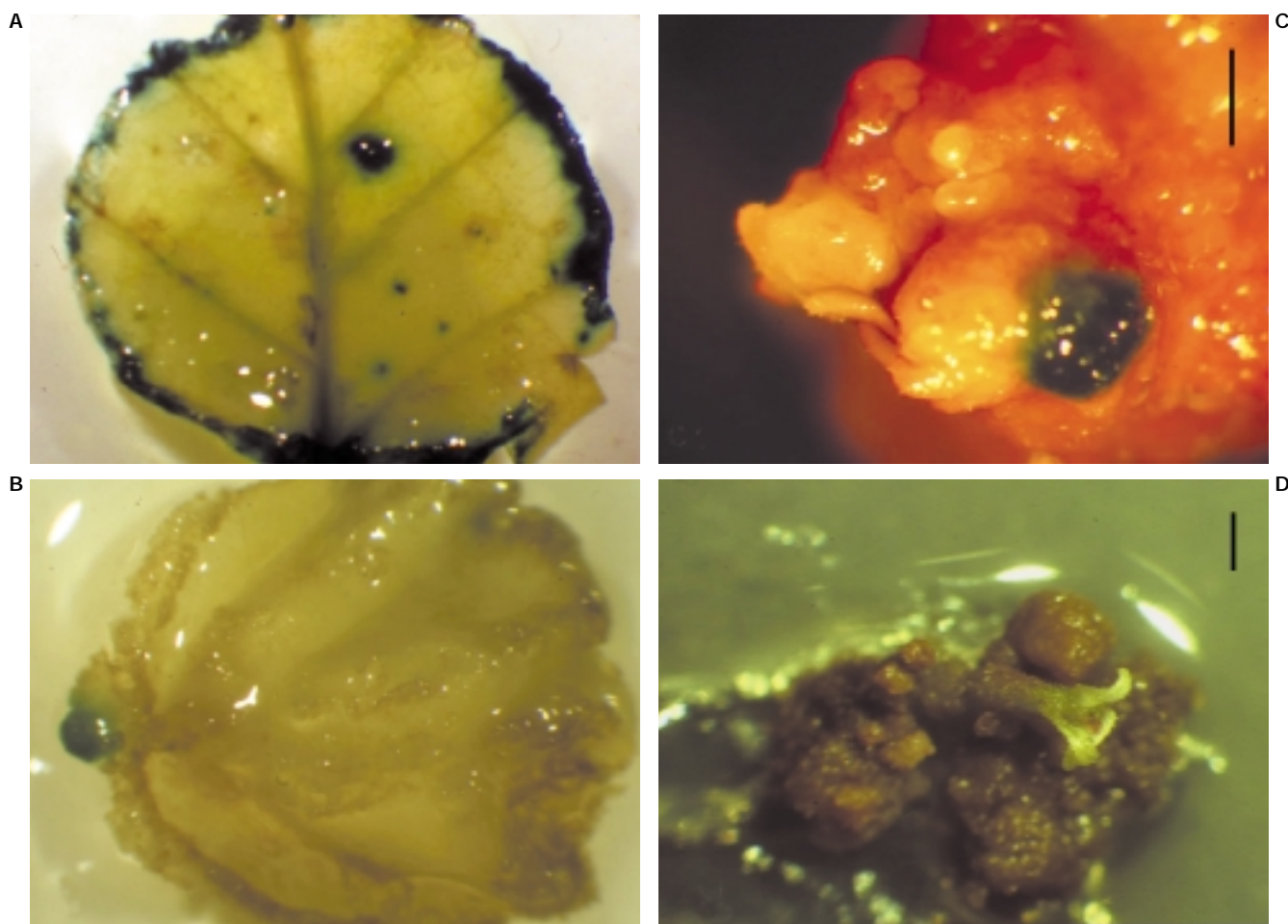


Figure 10. GUS expression (black dot) and regeneration, from leaf disc (8 mm diameter) of 'Sapporo' elm, after transformation experiment with *A. tumefaciens* GV3101-pMP90 (pKYGUSint.): A: after 1 week. B: after 3 weeks. C: after 5 weeks at the cut rib level (b: bud, m: meristem, c: callus, bar = 1mm). D: 'Sapporo' adventitious bud induced after 6 months on a selective medium containing 12.5 mg/l kanamycin. Bar = 1mm.

cess is well known for herbaceous species. On *Pisum sativum*, Loiseau *et al.* (1995) clearly showed the importance of fructose, maltose and sucrose on adventitious somatic embryo formation from apex, cotyledon and embryo axis. Maltose also stimulated somatic embryogenesis on alfalfa (Parrott and Bailey, 1993) and sugarcane (Brisibe *et al.*, 1994). The sugar effect is rather interesting; for 'Sapporo', buds were induced either by maltose 55mM and 110 mM or sorbitol 110mM, all other sugars being less efficient. We observed similar results with 'Commelin' except that, after 3 weeks, buds were still not induced with sorbitol (Ben Jouira *et al.*, 2000b). For 'Sapporo' and 'Commelin', buds induced on maltose can grow on maltose as well, provided reduced concentration ($\times \frac{1}{2}$) was applied.

In these experiments, 'Sapporo' shoots were easy to root. This result was quite surprising since rooting of *in vitro* mother plants needed an auxinic shock. This might be due to the nature of the original explant. For mother plants, the original explant is «cambium». It is presupposed to be rich in endogenous cytokinins since it naturally induces budding (Ben Jouira *et al.*, 1997). DKW macronutrients efficiency (Bolyard *et al.*, 1991; George and Tripepi, 1994) has been confirmed but only in the rooting medium. A genotype relationship was noted since DKW $\times \frac{1}{2}$ was optimum for 'Commelin' and DKW $\times \frac{1}{4}$ for 'Sapporo'.

We have shown previously the efficiency of wild strains of *Agrobacterium tumefaciens* (Ach5, C58) to induce tumors on 'Commelin' stem explants (Dorion *et al.*, 1995). We failed to observe «cambium» explants transformation with the same disarmed strains either because GUS expression was hidden by browning of the explants or because the activity of the promoter used (p 35S of CAMV) was not strong enough (unpublished results). When leaf discs were used in transformation experiments with an *Agrobacterium* strain containing the improved plasmid (pKYGUSint.), GUS expression was observed from the cocultivation until bud regeneration. After 5 weeks this expression can be considered as stable and interestingly, this took place in the leaf part showing regeneration ability. Unfortunately, 'Sapporo' leaf explants showed great susceptibility to neomycin selection at a low concentration as 12.5 mg/l, although other authors reported selection doses of 30 g/l kanamycin on *U. americana* (Bolyard *et al.*, 1991) and 50 g/l on *U. procera* (Fenning *et al.*, 1993). Nevertheless, such toxicity effects were reported for *Malus sp.* (Yepes and Aldwinckle, 1994). Therefore, if this work could be carried on, it should be necessary to determine the best

moment to apply the selection pressure or to choose other marker genes like hygromycin resistance.

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